DETERMINANTS OF β-ARRESTIN2 RECRUITMENT INTO PRIMARY CILIA

UNDERGRADUATE HONORS RESEARCH THESIS

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LIST OF ABBREVIATIONS

ACIII: adenylyl cyclase type III

BSA: bovine serum albumin

CTS: ciliary targeting sequence

FBS: fetal bovine serum

GFP: green fluorescent protein

GPCR: G protein-coupled receptor

GRK: G protein-coupled receptor kinase

IFT: intraflagellar transport

IMCD: inner medullary collecting duct

NES: nuclear export signal

NLS: nuclear localization signal

PBS: phosphate buffered saline

SST: somatostatin

SSTR: somatostatin receptor

Sstr3: somatostatin receptor 3

Veh: vehicle

βarr1: beta-arrestin1

βarr2: beta-arrestin2

ABSTRACT

Primary cilia are specialized organelles found on nearly every cell type in the human body. They perform specialized sensory and signaling functions, and are required for cellular homeostasis and proper development. Despite the fact that defects in primary cilia formation and function have been implicated in numerous diseases, the precise functions of primary cilia on most cell types are unknown. Primary cilia are known to coordinate signal transduction pathways, including G protein-coupled receptor (GPCR) signaling. However, little is known about the regulation of cilia-mediated GPCR signaling. β-arrestins are important regulators of GPCR signaling at the plasma membrane and act to terminate or facilitate GPCR signaling. Two different β-arrestin isoforms, β-arrestin1 (βarr1) and β-arrestin2 (βarr2), are expressed ubiquitously and regulate most GPCRs in a fairly nonspecific manner. Yet, we have discovered that agonist treatment initiates recruitment of βarr2, but not βarr1, into the ciliary compartment. We hypothesize that a specific domain(s) found in βarr2, mediates its recruitment into primary cilia following the activation of ciliary GPCRs. In order to identify the domain(s) mediating the recruitment of βarr2 into primary cilia, we used overlap extension PCR to generate chimeric proteins containing domains of βarr1 and βarr2. Our results indicate that two distinct domains within βarr2 are mediating its recruitment into the primary cilium following treatment with SST. Understanding the molecular basis of βarr2 recruitment into the primary cilium is a vital step to understanding the mechanism of ciliary GPCR signaling and its role in ciliary disorders.

INTRODUCTION

Cilia are classified as either motile or primary (Figure 1). The main function of motile cilia is the generation of flow or movement. Some examples of motile cilia include respiratory cilia, ependymal cilia, oviduct cilia, and sperm flagella. Primary cilia are immotile cellular appendages that mainly function as signaling and sensory organelles. These classifications are not mutually exclusive (Green and Mykytyn, 2010). Cilia are composed of an axoneme of nine doublet microtubules that extend from a basal body. The basal body is a specialized centriole that is linked to the plasma membrane by transition fibers. The structure of motile cilia is considered "9+2" due to the nine doublet microtubules that surround two central microtubules (Figure 2). The structure of primary cilia is considered "9+0" due to nine doublet microtubules and the absence of central microtubules (Satir et al., 2010). There are some exceptions to these designations including the "9+0" motile cilia on the embryonic node and the "9+2" immotile cilia on olfactory neurons (Green and Mykytyn, 2010).

The primary cilium is a cellular appendage that is found on nearly every cell type in the human body. The primary cilium can be thought of as a "cellular antenna" which performs specific sensory and signaling functions for the cell. The main function of primary cilia is to transduce signals from extracellular stimuli to a cellular response (Basten and Giles, 2013) Primary cilia are essential for proper embryonic development. Signaling cascades through the primary cilia are responsible for tissue homeostasis, specification of the plane of the cell, and apoptosis. Primary cilia are also able to respond to mechanical stimulation and chemosensation (Satir et al., 2010). The signaling functions of primary cilia are determined by the specific proteins that are present in the ciliary compartment (Green and Mykytyn, 2010).

Proteins needed for the formation and function of primary cilia must be transported from the cell body into the ciliary space due to the fact that the ciliary compartment lacks the machinery to synthesize proteins. The transition fibers at the base of the cilium act to allow only

certain proteins to enter the ciliary space (Green and Mykytyn, 2010). In order to form a primary cilium, the centrosome migrates to the cell surface during the G_0 phase of the cell cycle. The mother centriole attaches to a Golgi derived vesicle and expands with the axoneme growing above the centriole within the ciliary membrane. The growing ciliary membrane is continuous with the plasma membrane (Satir et al., 2010).

Intraflagellar transport (IFT) is essential for moving the proteins needed for the formation and function of primary cilia (Satir et al., 2010). IFT is a highly conserved transport mechanism which bi-directionally moves proteins in the ciliary compartment. Proteins that will be moved into the ciliary compartment are transported in vesicles from the Golgi-apparatus to the base of the cilium where they are exocytosed and attached to IFT particles (Figure 3). Following the protein's association with IFT particles, the complex is transported via anterograde IFT to the distal cilia tip by kinesin-2 motors where the proteins are unloaded. The IFT particles which have unloaded their proteins are then transported via retrograde IFT to the cell body by cytoplasmic dynein 2 (Petersen and Rosenbaum, 2008). IFT is essential for the formation and function of primary cilia and defects in IFT lead to severe disease and developmental defects (Green and Mykytyn, 2010).

Proteins that are destined for the primary cilia membrane contain targeting sequences which allow the protein to efficiently localize to the primary cilium. Several ciliary targeting sequences (CTS) have been identified. Rhodopsin contains a C-terminal CTS with a VxPx motif. Polycystin-2 has an N-terminal RVxP motif. Polycystin-1 contains a C-terminal KVHPSST motif. These studies have led to the possibility of a generic CTS, a VxP motif (Hsiao, et al., 2012). This generic motif does not cover all possible CTSs. The third intracellular loop of GPCRs has been shown to possess a CTS for receptors that are localized to the ciliary membrane. Sstr3, Htr6, and Mchr1 are all ciliary GPCRs. Each of these receptors expresses an Ax(S/A)xQ motif in their third intracellular loop signaling the possibility of an additional CTS

(Berbari, et al., 2008). Many proteins that are targeted to the primary cilium do not possess a known CTS which leads to the possibility of identifying additional CTSs.

G protein-coupled receptors are transmembrane receptor proteins that have been shown to regulate numerous signaling pathways. These signaling pathways include cellular metabolism, secretion of cellular components, cell growth and division, immune responses and sensory signaling pathways involving smell, taste and vision. GPCRs are activated by binding of their ligand. Following the binding of a ligand the intracellular domain of the GPCR undergoes a conformational change. Activated receptors can activate their associated G proteins by exchanging a GDP for a GTP. Once the G protein has been activated the G protein's α subunit with the bound GTP can disassociate from the β and γ subunits to affect intracellular signaling (Figure 4). There are over 800 known GPCRs in the human genome, and GPCRs regulate some aspect of all most every physiological function (Premont and Gainetdinov, 2007).

Only certain GPCRs are able to localize to the ciliary membrane, as demonstrated by the somatostatin receptors. Five somatostatin receptors (SSTRs) are present in the human body. The SSTR family is activated by the ligand somatostatin (SST). SST is expressed ubiquitously throughout the human body. SST functions as an inhibitory peptide for endocrine and exocrine function throughout the body; its effects include inhibiting hormone secretion, inhibiting cell proliferation, and promoting apoptosis (Figure 5) (Barbieri, et al., 2013). Although there are five SSTRs present in humans, only somatostatin receptor 3 (Sstr3) is targeted to the ciliary membrane (Berbari et al., 2008) (Figure 6). The Sstr3 receptor is critical for object recognition memory in mice, suggesting that Sstr3 on primary cilia of central neurons may be essential for memory function (Einstein et al., 2010).

β-arrestins are expressed ubiquitously in all cells and tissues, and function as regulators of GPCR signaling pathways and also serve as multi-functional adaptors and signal

transducers. β-arrestins terminate GPCR signaling pathways by acting as adaptor proteins to form complexes with GPCRs (DeWire, et al., 2007). Following the activation of a GPCR by its ligand, the receptor is phosphorylated by G protein-coupled receptor kinases (GRKs). After phosphorylation, β-arrestins are recruited to the receptor which targets them for clathrin mediated endocytosis (Figure 7) (Luttrell and Lefkowitz, 2002). Two β-arrestins have been identified, β-arrestin1 (βarr1) and β-arrestin2 (βarr2). The amino acid sequences of the two proteins are 78% identical, and most of the coding differences appear in the C terminus (Figure 8) (DeWire et al., 2007). While the proteins are similar there are some key differences in their cellular localization. βarr1 is localized to both the cytoplasm and the nucleus due to a nuclear localization sequence (NLS) (Hoeppner, 2012). βarr2, however, is excluded from the nucleus and is localized to the cytoplasm due to a nuclear export signal (NES) in its C-terminus (Figure 9) (Wang, et al., 2002).

The two β-arrestin isoforms have been shown to have similar functions (Luttrell and Lefkowitz, 2002). A previous study has found that a knockout animal for both β-arrestin proteins is embryonic lethal (Kovacs et al., 2009). Post-development there is still some overlap between the β-arrestin isoforms, but there are also some GPCRs where the internalization is mediated primarily by one isoform. For example the internalization of β-2 adrenergic receptor is mediated primarily by βarr2 (DeWire, et al., 2007).

Defects in primary cilia formation and function have been implicated in disease pathogenesis; these diseases are collectively called ciliopathies. Ciliopathies range in prevalence from 1 in 1,000 to 1 in 150,000. As primary cilia are found on nearly every cell type in the human body, defects in primary cilia affect many parts of the body. Ciliopathies cause multisystem pathology, and their effects are devastating. There are no cures and very few treatments for ciliopathies. Some ciliopathies include Bardet-Biedl Syndrome, Meckel Syndrome, Joubert Syndrome, and polycystic kidney disease. Examples of disease phenotypes caused by defects in primary cilia include renal cystic disease, retinal degeneration, situs

inversus, cognitive defects, and obesity (Figure 10). Although ciliopathies may be statistically rare, the disease phenotypes which are associated with ciliopathies are very common (Tobin and Beales, 2009).

Sstr3 is a ciliary GPCR that is activated by the binding of its ligand somatostatin (SST). Upon treatment with SST, βarr2 is recruited into primary cilia. We suspect that βarr2 interacts with Sstr3 and is involved with the termination of its signaling. βarr1 however, is not recruited into primary cilia (Figure 9). This difference in recruitment suggests that specific amino acids within βarr2 allow it to pass the transition fiber and are mediating its recruitment into the primary cilium to interact with ciliary receptors. The amino acid sequences of these two proteins are 78% homologous. We hypothesize that specific domain(s) found in βarr2, mediate its recruitment into primary cilia following treatment with SST. The domain(s) within βarr2 that mediate its recruitment to primary cilia will be identified through the creation of chimeric proteins using overlap extension PCR. The chimeric proteins will be ligated into a mammalian expression vector containing a green fluorescent protein (GFP) tag, and then co-transfected into a ciliated cell line (IMCD cells) with Sstr3. Following transfection, the cells will be treated with SST to activate Sstr3. Wild type βarr1 and βarr2 will act as controls for the SST treated and vehicle conditions. Immunocytochemistry will be performed, and the cells will be visualized using a confocal microscope. These results will allow us to further narrow down domains necessary for the recruitment of βarr2 into primary cilia in order to select amino acids of interest.

Overall, primary cilia are sensory organelles that coordinate numerous different signal transduction pathways, including GPCR signaling. Despite the fact that GPCR dysfunction has been implicated in disease pathways throughout the body, little is known about the regulation of cilia-mediated GPCR signaling and the effects on cellular function. We have discovered that treatment of cells with the ligand for a GPCR on the ciliary membrane of central neurons initiates recruitment of βarr2 into the ciliary compartment. β-arrestins are important regulators of

GPCR signaling pathways and also serve as multi-functional adaptors and signal transducers (Whalen et al., 2011). Thus, understanding the molecular basis of βarr2 recruitment into the primary cilium is a vital step to understanding the mechanism of ciliary GPCR signaling and may lead to therapeutic interventions for ciliopathies and other diseases.

MATERIALS AND METHODS

Plasmid Construction

The coding sequence of mouse Sstr3 was subcloned into pcDNA3.1/myc-His (Life Technologies/Invitrogen) vector. The coding sequences of mouse βarr1 and βarr2 were subcloned into pEGFP-N (Clontech) vector. All DNA sequences were verified at the Nuclei Acid Shared Resource at The Ohio State University.

Chimeric Protein Construction

The sequences of interest of the β-arrestins were amplified from mouse DNA constructs, and then fused together to create a chimeric protein made of part of βarr1 and part of βarr2 using overlap extension PCR (Vallejo et al., 2008). Oligonucleotide primers for the nucleotide sequence of the junction regions were created. The primers contained a 5' nucleotide sequence from one β-arrestin and a 3' nucleotide sequence from the other β-arrestin at the junction point. The junction points were spanned by conserved residues and maintained the coding frame. Primers for the N-terminal and C-terminal regions were designed in order to add a restriction site for cloning. A HindIII restriction site was added to the N-terminus, and a KpnI restriction site was added to the C-terminus. The initial PCR fragments of the two β-arrestin fragments were gel purified and combined to be used as template for the fusion PCR. During the fusion PCR the complementary overhanging sequences allowed for the creation of one continuous chimeric protein that also contained the desired restriction sites. The final PCR fragments were cloned into pSTBlue-1(Novagen/EMDMillipore), a TA cloning vector, digested out of the vector using the inserted restriction sites, and then cloned into the pEGFP-N (Clontech) mammalian expression vector. The sequences of the chimeric proteins were verified through the Nucleic Acid Core at the Ohio State University.

Cell Culture and Treatment

IMCD cells (ATCC) were cultured in DMEM:F12 media supplemented with 10% FBS, sodium bicarbonate, and sodium pyruvate. Cells were co-transfected using electroporation with 10μg of DNA (5μg of pEGFP-N- βarr chimera and 5μg of pcDNA3.1-myc/his-Sstr3) and plated on glass coverslips. For each chimera experiment wild type pEGFP-N-βarr1 and βarr 2 were also co-transfected with pcDNA3.1-myc/his-Sstr3 to act as controls. Cells were given fresh media at 24h post transfection. Cells were treated at 48h post-transfection. The glass coverslips which the cells had been grown on were placed in individual wells with 1mL of fresh media. Somatostain (Phoenix Pharmaceuticals; #060-03) was suspended in water for a concentration of 1mM. Cells were treated with 10μL water (vehicle condition) or 10μL of somatostatin (treated condition) for 20min at 37°C.

Immunofluorescence

Cells were fixed in 4% paraformaldehyde for 15min and permeabilized with 0.3% Triton X-100 in PBS with 2% goat serum, 0.02% sodium azide, and 1 g/mL bovine serum albumin (BSA) for 10min. The cells were labeled with mouse anti-myc primary antibody (1:400 concentration; Santa Cruz Biotechnology) for 16-24h at 4°C, and Alexa Fluor 546-conjugated goat anti-mouse IgG secondary antibody (1:1000 concentration; Life Technologies/Molecular Probes) for 1h at room temperature. Nuclei were labeled with DRAQ5 (1:2500 concentration; Cell Signaling). All incubations and washes were carried out in PBS with 2% goat serum, 0.02% sodium azide, and 1 g/mL BSA. Slides were mounted using Immu-Mount.

Microscopy

Cells were imaged using a Zeiss LSM 510 laser scanning confocal microscope at The Ohio State Curtis-Hunt Microscopy Imaging Facility in the Department of Neuroscience at The Ohio State University. Recruitment of chimeric β-arrestins was compared to the recruitment of the controls (βarr1 and βarr2) in order to identify which of the chimeras were recruited into the primary cilium following treatment with SST. Recruitment was identified by observing a co-localization of β-arrestin and Sstr3 in the primary cilium following SST treatment.

RESULTS

We have previously shown that βarr2 is recruited into primary cilia in response to SST treatment. βarr1, on the other hand, is not recruited into primary cilia. We hypothesize that this difference is due to a specific domain(s) within βarr2 mediating its ciliary recruitment following treatment with SST. In order to identify the domain(s) of βarr2 mediating ciliary recruitment we constructed chimeric proteins containing regions of βarr1 and βarr2 through overlap extension PCR and tested whether the chimeras were recruited into cilia in response to SST (Figure 11).

To determine whether sequences within the N-terminal or C-terminal half of βarr2 were mediating recruitment, we created chimeras in which the N-terminal half of βarr1 was fused to the C-terminal half of βarr2 (chimera 1), and the N-terminal half of βarr2 was fused to the C-terminal half of βarr1 (chimera 2). Following treatment with SST chimera 1, but not chimera 2, was recruited into cilia (Figure 12). Suggesting that the C-terminal half of βarr2 is mediating its recruitment into cilia following SST treatment, and narrowing the region of interest to amino acids 203-410 of βarr2.

In order to further narrow down the domain of interest, chimeras that further divided the C-terminal half of βarr2 into smaller domains were constructed. Chimera 3 contained amino acids 1-341 of βarr2 followed by amino acids 347-418 of βarr1. Chimera 4 contained amino acids 1-347 of βarr1 and amino acids 341-410 of βarr2. Interestingly, both chimera 3 and chimera 4 were recruited to cilia following SST treatment, suggesting that two domains in the C-terminal half of βarr2 are capable of mediating ciliary recruitment.

To further narrow down the domain of interest indicated by chimera 4 (amino acids 341-410 of βarr2), we constructed chimera 5, which contained amino acids 341-372 of βarr2, and chimera 6, which contained amino acids 373-410 of βarr2. Chimera 6, but not chimera 5, was recruited to cilia following SST treatment, thereby narrowing one region of

interest to amino acids 373-410 of βarr2. Thus, the C-terminal 38 amino acids of βarr2 are sufficient to confer SST-mediated ciliary recruitment to βarr1.

To further narrow down the domain of interest indicated by chimera 3 (amino acids 203-341 of βarr2), and to confirm the presence of two distinct domains capable of mediating ciliary recruitment, we constructed chimera 7 containing amino acids 270-341 of βarr2. Following SST treatment, chimera 7 was recruited into primary cilia. This result confirms that there are two separate domains in βarr2 that are individually capable of mediating ciliary recruitment. In summary we have identified two distinct domains in βarr2 (amino acids 270-341 and 373-410) that mediate ciliary recruitment.

DISCUSSION

Primary cilia are essential for cell communication and signaling. GPCRs, such as Sstr3, are selectively targeted to the ciliary membrane (Green and Mykytyn, 2010). β-arrestins are known regulators of GPCR signaling on the plasma membrane (Pierce and Lefkowitz, 2001). This suggests a possible role for β-arrestins as regulators of GPCRs on the ciliary membrane. Through investigating the role of β-arrestins in regulating ciliary GPCRs, we have shown that only βarr2 is recruited to the primary cilium following SST treatment.

In order to provide mechanistic insight into βarr2 ciliary recruitment, we sought to identify the domain(s) of βarr2 that is responsible for its recruitment into primary cilia. Through the creation of chimeric proteins, we have shown that there are two distinct domains in the C-terminal half of βarr2 that can mediate ciliary recruitment. These domains are between amino acids 270-341 and amino acids 373-410 of βarr2. We have demonstrated that either of these distinct domains are sufficient to confer ciliary recruitment to βarr1.

Future experiments utilizing additional chimeric proteins will be aimed at further narrowing these domains of interest, in order to more fully understand the recruitment of βarr2 to primary cilia. The domain of interest consisting of amino acids 270-341of βarr2 contains 13 different amino acids when compared to wild type βarr1. Within this region, βarr1 also contains 8 additional amino acids that are not found in βarr2 (amino acids 333-340). Once the domains have been sufficiently narrowed to a point where there are only several amino acids that are different between the sequences of βarr1 and βarr2, we will perform site-directed mutagenesis in order to mutate wild type βarr1 to contain the suspected amino acids of βarr2. We will then test whether ciliary recruitment is affected following the insertion of the 8 amino acid sequence of βarr1 into wild type βarr2 (amino acids 333-340).

The domain of interest between amino acids 373-410 of βarr2 contains 6 different amino acids, as compared to βarr1, that are possible targets for site-directed mutagenesis. βarr2 also

contains a 3 amino acid YAT (tyrosine - alanine - threonine) sequence (amino acids 381-383) that is not found in βarr1. This 3 amino acid sequence could be inserted into wild type βarr1 to test whether it can confer ciliary recruitment. This domain of interest also contains the NES that is responsible for the exclusion of βarr2 from the nucleus, it would be interesting to test whether the presence of a NES would confer ciliary recruitment to βarr1.

Once we have identified the amino acids that mediate βarr2 ciliary recruitment, it would be interesting to determine whether that sequence is sufficient to mediate the recruitment of other proteins into the primary cilia. This could be accomplished through the fusion of the identified amino acid sequence to GFP. The recruitment of the chimeric protein after treatment with SST could then be assessed in order to determine whether the sequence is a possible motif that may aid in the identification of additional proteins that are recruited to primary cilia.

Additionally, it would be interesting to determine which proteins βarr2 is interacting with once it has entered the ciliary compartment. Through utilizing a yeast two-hybrid system, it would be possible to not only identify interactions of βarr2 with known ciliary proteins, but also its potential interactions with ciliary proteins that have yet to be identified (Miller and Stagljar, 2004). Understanding additional protein interactions may aid in understanding the function of βarr2 within the primary cilium. We suspect that upon recruitment, βarr2 binds to Sstr3, which has been activated by treatment with SST, and mediates its translocation out of the cilium. The effect this has on Sstr3 signaling is unknown. Similar to the role of β-arrestins on the plasma membrane (Pierce and Lefkowitz, 2001), βarr2 may be acting to terminate Sstr3 signaling on the ciliary membrane . It is also possible that βarr2 is acting as a signal transducer molecule in a GPCR signaling cascade, as evidence has suggested that β-arrestins have the capability to function as scaffolds for different signaling cascades (Kovacs et al., 2009).

Understanding the mechanism by which βarr2 is transported into the ciliary compartment will also give insight into the overall function of βarr2 in primary cilia. It is possible that βarr2 may be transported through IFT motors, or through lateral diffusion in the ciliary

membrane. We will investigate these possible methods by treatment with various drugs. For example the lateral fluidity of the ciliary membrane can be inhibited through treatment with WGA. This will allow for the identification of the transportation method responsible for the recruitment of βarr2.

The ciliary space is distinct from the rest of the cell, and trafficking to and from the primary cilium is highly regulated (Hsiao et al., 2012). Understanding the molecular basis of the recruitment of βarr2 to primary cilia following SST treatment, and suspected ciliary receptor activation, is a vital step to understanding the mechanism that allows certain proteins to enter the ciliary space while excluding others. Ciliary signaling relies on the transport of proteins into and out of the primary cilium, therefore it is essential to understand the mechanism by which βarr2 is transported into the primary cilium. Furthermore, GPCRs are frequent targets of drug therapies, and further understanding the signaling capability of GPCRs and their possible regulation through molecules such as β-arrestins will be essential in developing future therapies for many diseases including ciliopathies (Whalen et al., 2011). The identification of the amino acid sequence(s) that is mediating the recruitment of βarr2 into the primary cilium will be a vital part of the understanding of the overall mechanism of its recruitment and interaction with GPCRs on the ciliary membrane. We have shown that there are two distinct domains within the C-terminal half of βarr2 that each are capable of individually mediating its recruitment to primary cilia following treatment with SST. This knowledge will aid in the understanding of the overall mechanism of ciliary recruitment and may offer insight for treatments of ciliopathies and other diseases which result from dysfunctional ciliary signaling.

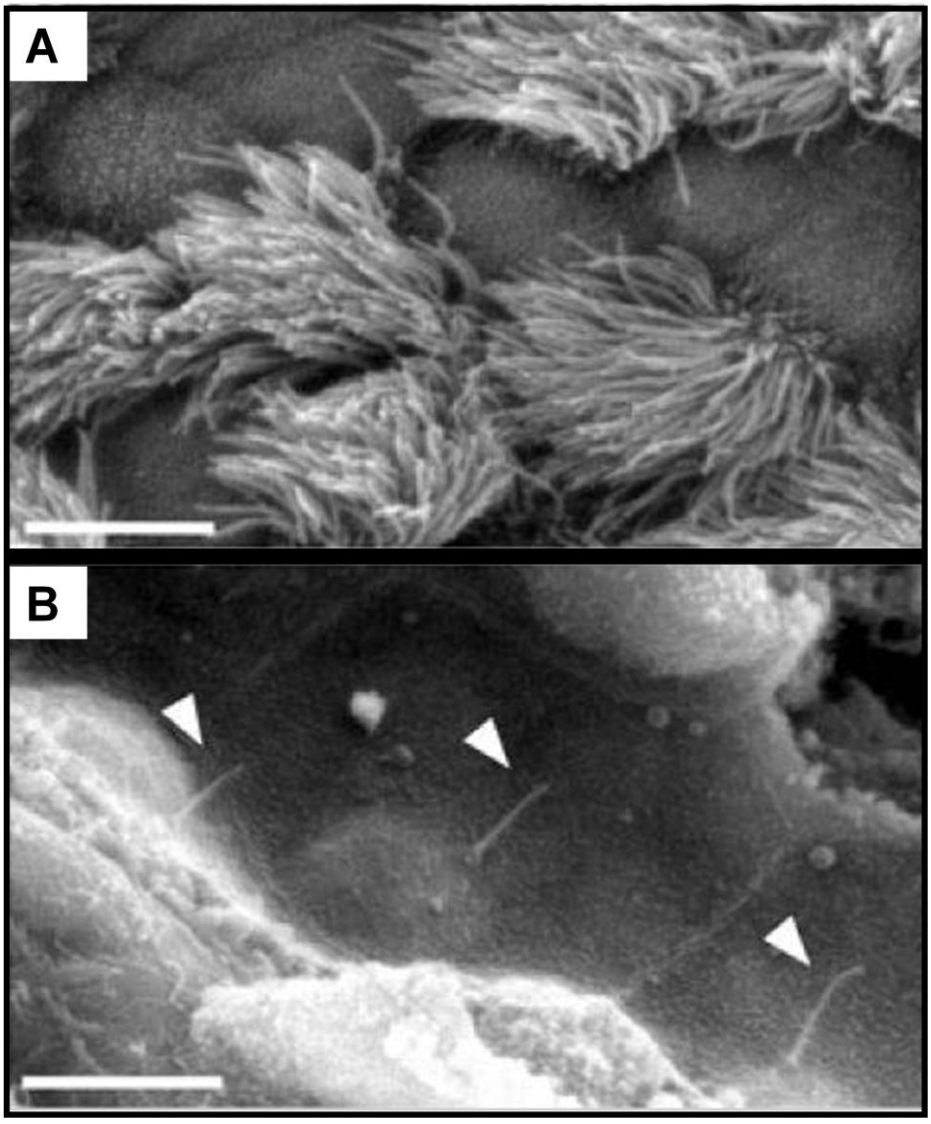

Figure 1 Motile vs Primary Cilia.

There are two types of cilia, motile (A) and primary (B). The main function of motile cilia is the generation of flow or movement. Primary cilia mainly function as signaling and sensory organelles. These classifications are not mutually exclusive (Green and Mykytyn, 2010).

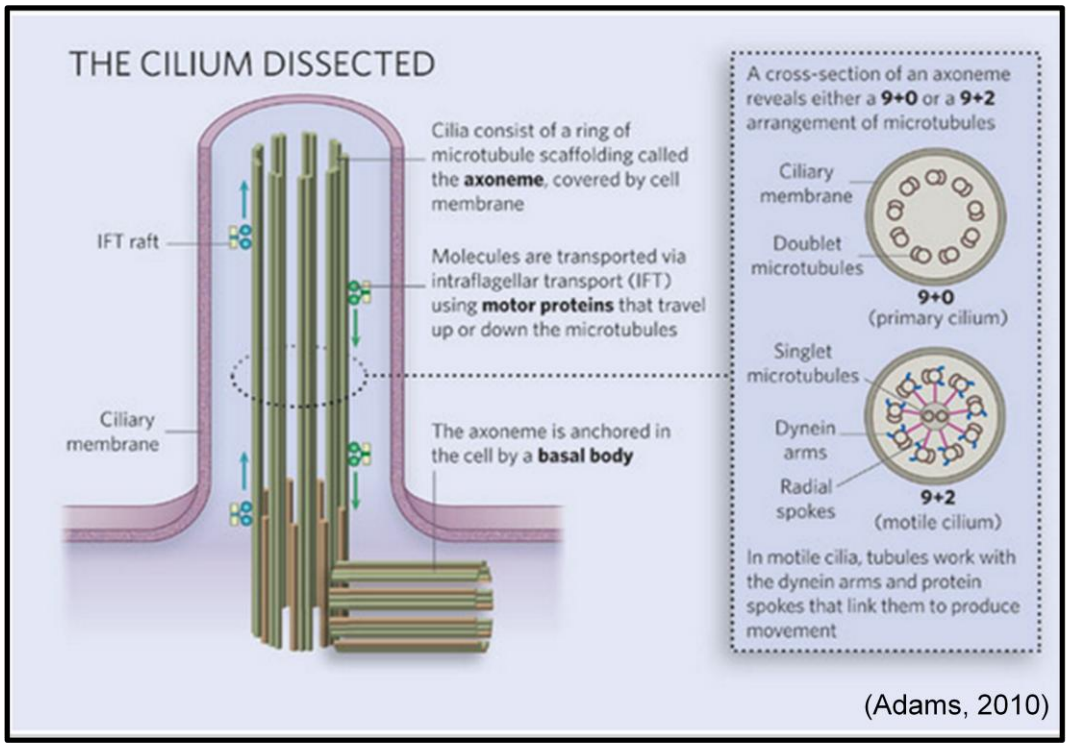

Figure 2 The Cilium Dissected.

Cilia are composed of an axoneme of nine doublet microtubules that extend from a basal body. The basal body is a specialized centriole that is linked to the plasma membrane by transition fibers. The structure of motile cilia is considered "9+2" due to the nine doublet microtubules that surround two central microtubules. The structure of primary cilia is considered "9+0" due to nine doublet microtubules, there are no central microtubules (Satir, 2010) (Adams, 2010).

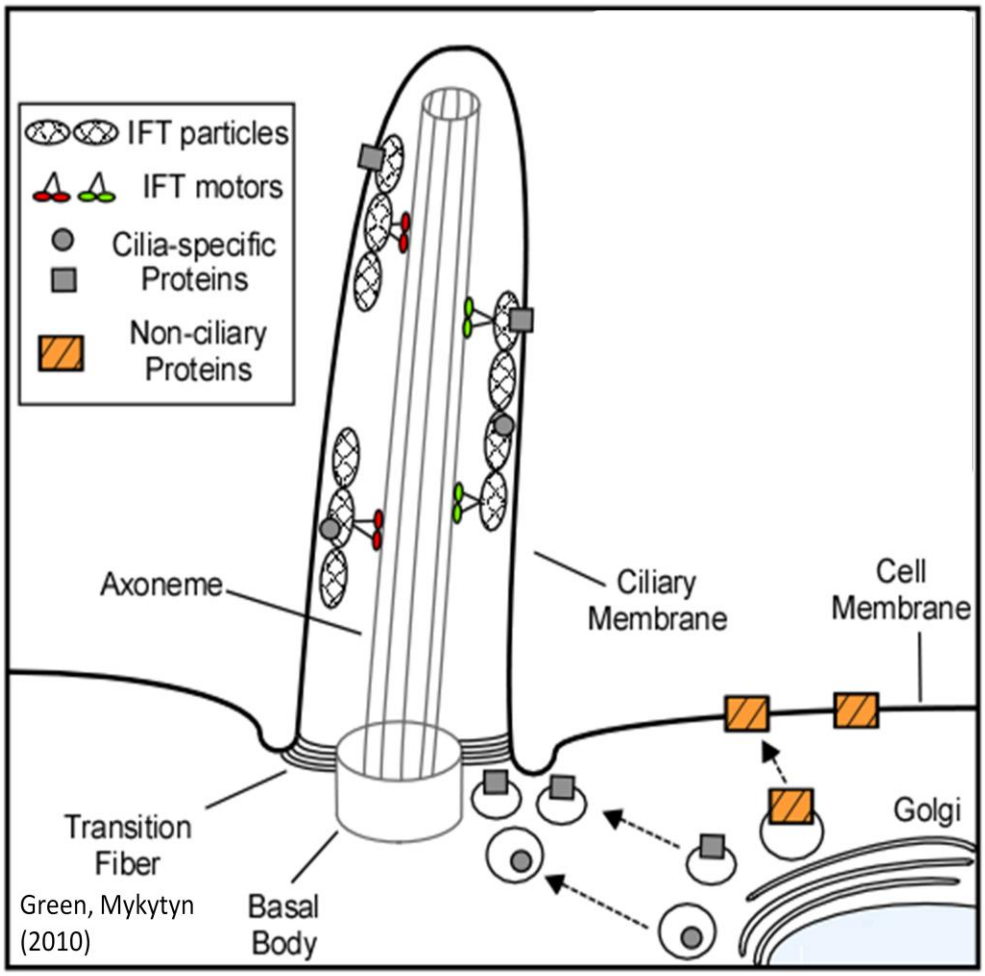

Figure 3 Schematic of primary cilia structure.

The ciliary space is separated from the intracellular space by the transition fiber at its base which acts as a selective membrane to only allow certain proteins to enter and exit the cilium. Proteins are exported from the Golgi to the base of the primary cilium where they associate with intraflagellar transport (IFT) particles and motors and then are selectively transported along the axoneme into the ciliary space (Green and Mykytyn 2010).

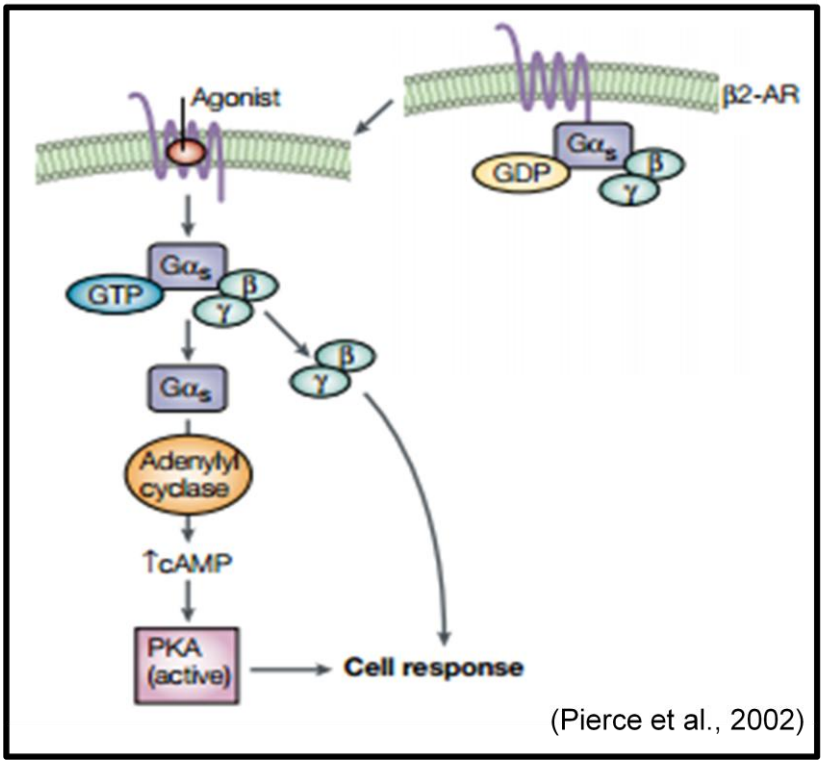

Figure 4 GPCR Signaling Cascade.

G protein-coupled receptors are transmembrane receptor proteins that have been shown to regulate numerous signaling pathways. GPCRs are activated by binding of their ligand. Following the binding of a ligand the intracellular domain of the GPCR undergoes a conformational change. Activated receptors can activate their associated G proteins by exchanging a GDP for a GTP. Once the G protein has been activated the G protein's α subunit with the bound GTP can disassociate from the β and γ subunits to affect intracellular signaling (Pierce et al., 2002).

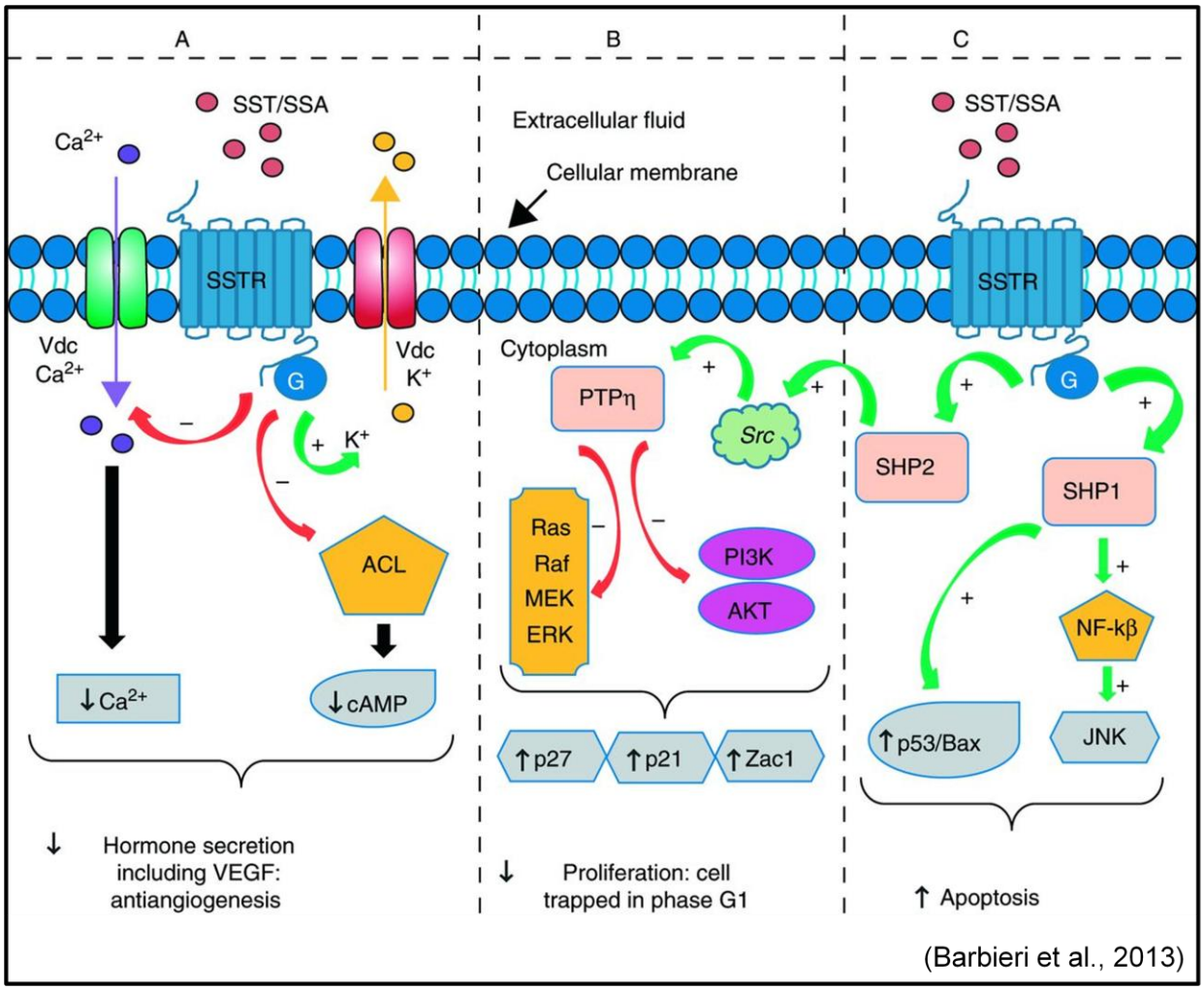

Figure 5 Functions of Somatostatin Receptor Family.

There are five somatostatin family receptors (SSTRs). The SSTRs are activated by the binding of somatostatin (SST). SST functions as an inhibitory peptide for endocrine and exocrine function throughout the body; its effects include inhibiting hormone secretion, inhibiting cell proliferation, and promoting apoptosis (Barbieri, et al., 2013).

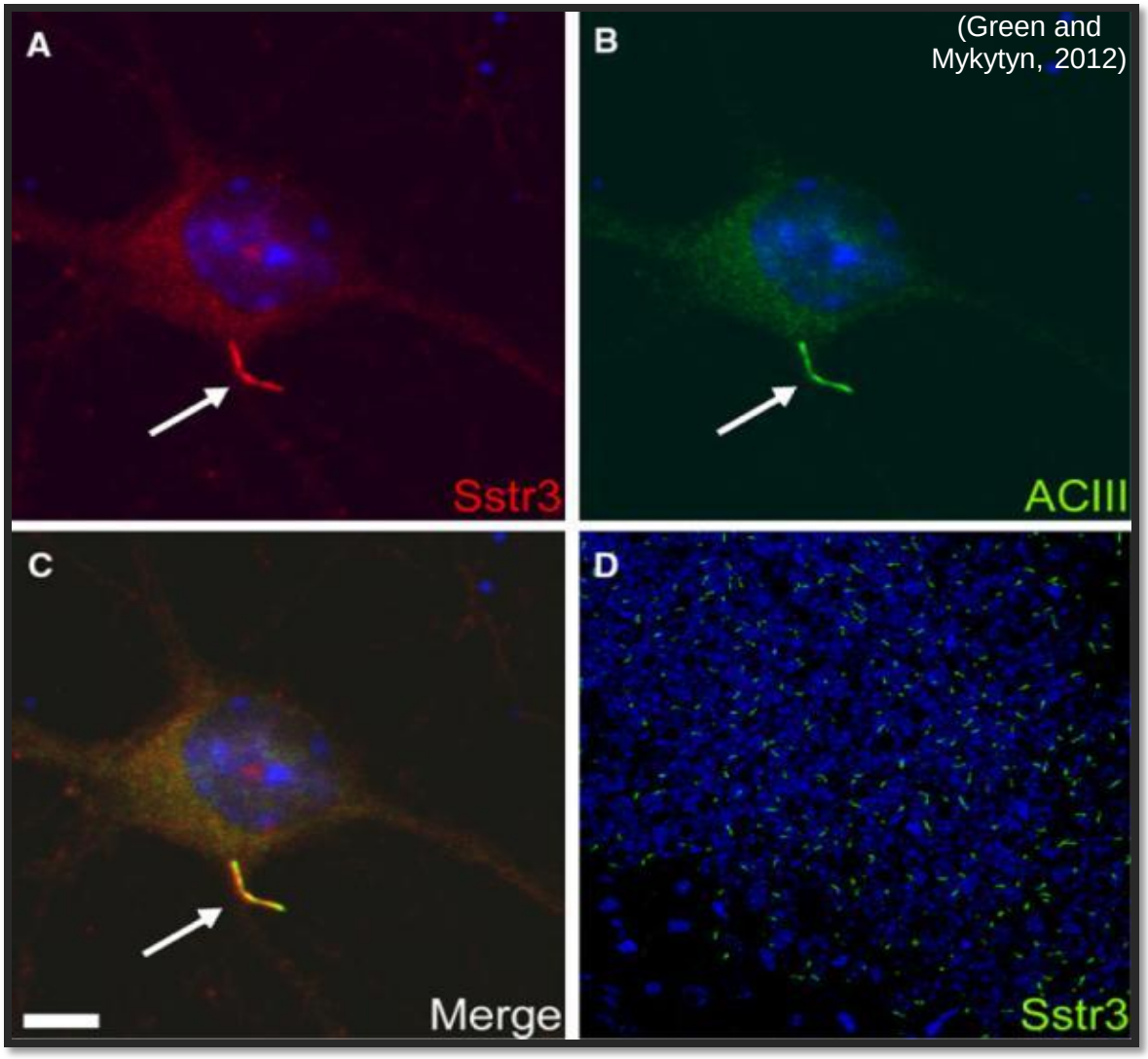

Figure 6 Somatostatin Receptor 3 Ciliary Localization.

Panels A-C: Cultured neurons labeled with anti-Sstr3 (somatostatin receptor 3) and anti-ACIII (adenylyl cyclase type III), a neuronal ciliary marker. Co-localization indicates that the GPCR Sstr3 localizes to neuronal cilia. Panel D: Brain section showing the abundance of Sstr3 positive primary cilia. Scale bar = 5 µm.

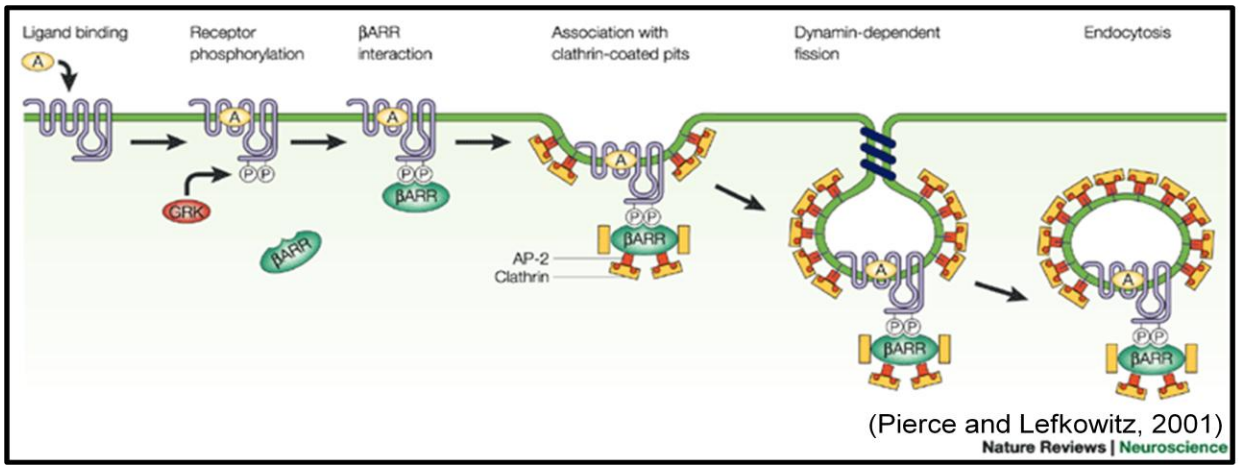

Figure 7 Schematic of β-arrestin mediated endocytosis.

Following the activation of the receptor by its ligand G-protein-coupled receptor kinases (GRKs) phosphorylate the receptor. β-arrestin then interacts with the phosphorylated receptor causing the creation of a clathrin coated pit. The clathrin coated pit buds into the cell through dynamin dependent fission, and eventually the vesicle is fully engulfed and dissociates from the membrane (Pierce and Lefkowitz, 2001).

Figure 8 BLAST Sequence Comparison of βarr1 vs βarr2.

BLAST comparison of the amino acid sequences of βarr1 and βarr2. The sequences of these 2 mouse isoforms are 87% homologous. A nuclear localization signal (NLS) is located in βarr1 between amino acids 157-170. A nuclear export signal is present in βarr2 between amino acids 388-397. Arrows denote fusion points of chimeric proteins. The red arrow denotes the fusion point for chimeras 1 and 2. The blue arrow denotes the fusion point for chimeras 3 and 4. The included region of βarr2 in chimera 5 is between the blue and orange arrows. The orange arrow denotes the fusion point of chimera 6. The included region of βarr2 in chimera 7 is between the green and blue arrows.

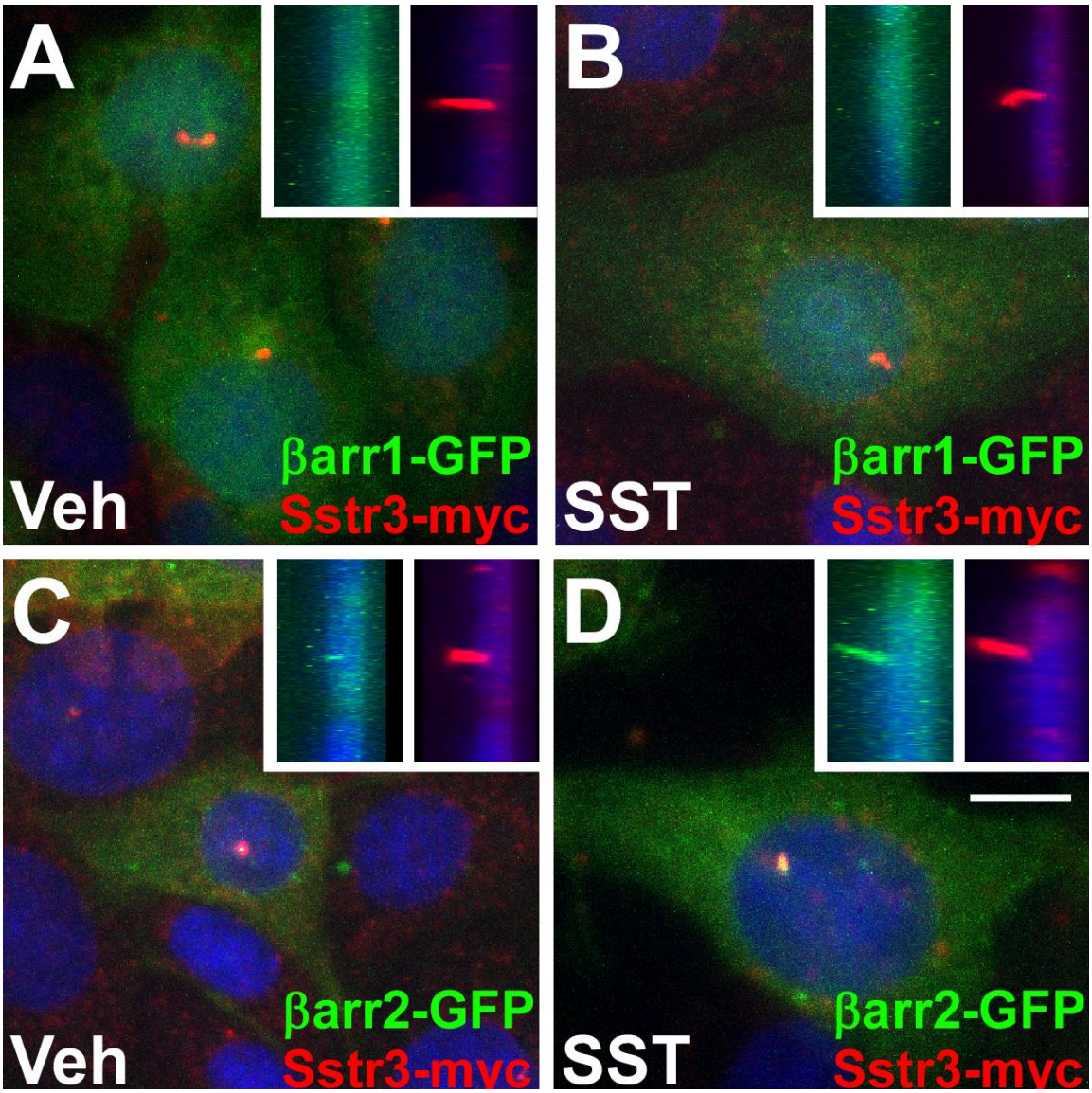

Figure 9 Ciliary Recruitment of βarr1 vs βarr2.

IMCD cells co-transfected with βarr1-GFP or βarr2-GFP and Sstr3-myc. Cells were vehicle (Veh) or somatostatin (SST) treated. Only βarr2 is recruited into the primary cilium following Sstr3 activation. Scale bar = 5 µm.

Feature	BBS	MKS	JBTS	NPHP	SLSN	JATD	OFD1	EVC	ALMS	PKD
Renal cysts	√	√	√	√	√	√	√			√
Hepatobiliary disease	√	√	√	√	√	√	√		√	√
Laterality defect	√	√		√		√				
Polydactyly	√	√	√			√	√	√		
Agenesis of corpus callosum	√	√	√			√	√			
Cognitive impairment	√	√	√			√	√	√		
Retinal degeneration	√	√	√		√	√			√	
Posterior fossa defects/encephalocoele	√	√	√			√		√		
Skeletal bone defects						√	√	√		
Obesity	√								√	

BBS, Bardet-Biedl syndrome; MKS, Meckel syndrome; JBTS, Joubert syndrome; NPHP, Nephrophthisis; SLSN, Senior-Løken syndrome; JATD, Jeune syndrome; OFD1, Oro-facial-digital syndrome type 1; EVC, Ellis van Creveld syndrome; ALMS, Alström Syndrome; PKD, polycystic kidney disease.

Figure 10 Ciliopathies and their Symptoms.

Defects in primary cilia formation and function can lead to a wide range of disease phenotypes collectively known as ciliopathies. These diseases affect multiple body systems (Tobin and Beales, 2009).

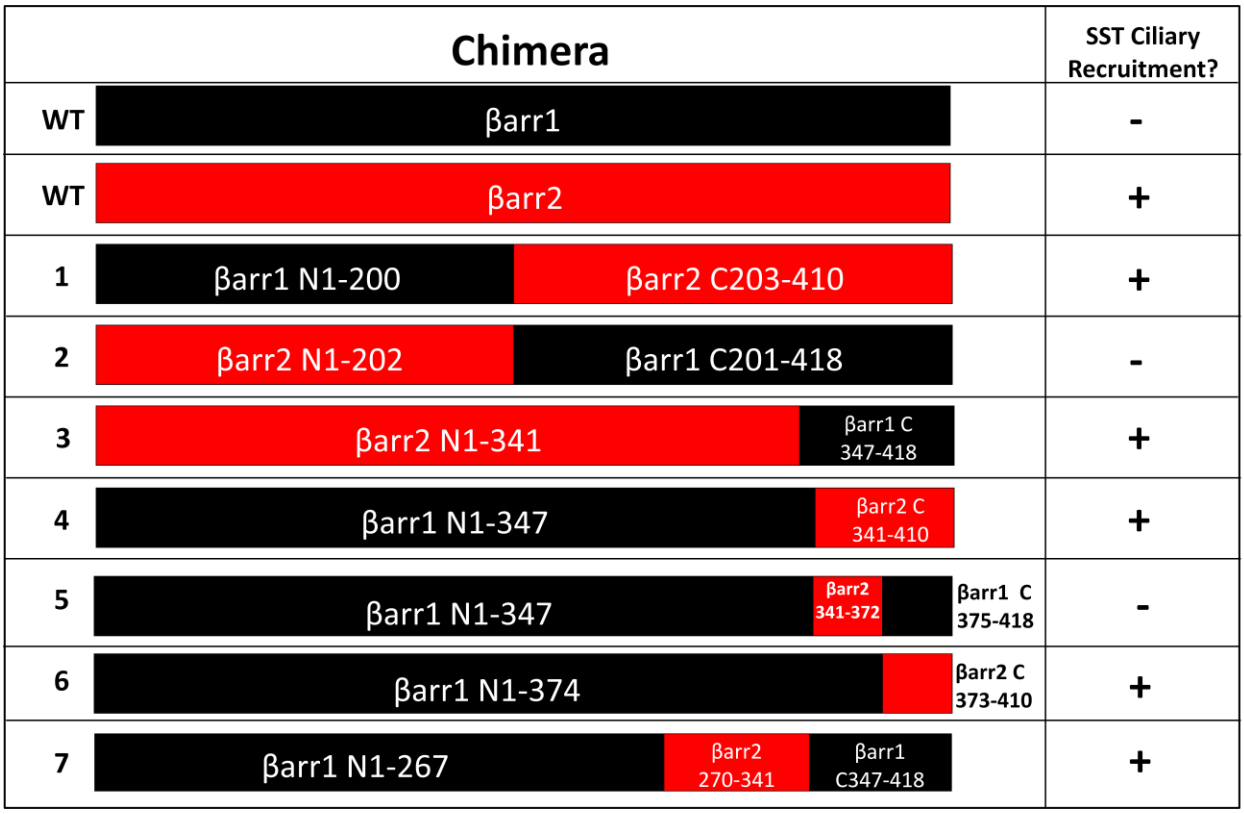

Figure 11 Chimera Recruitment.

Chimeric proteins created via overlap-extension PCR. Chimeric proteins were fused to a GFP construct. βarr constructs were co-transfected with Sstr3-myc into IMCD cells. Confocal microscopy was utilized in order to examine localization of chimeric protein constructs in vehicle and somatostatin treated conditions. There are two domains of βarr2 that mediate its recruitment following ciliary receptor activation. The first domain is present within the amino acids 270-341 of βarr2, and the second is within the amino acids 373-410 of βarr2.

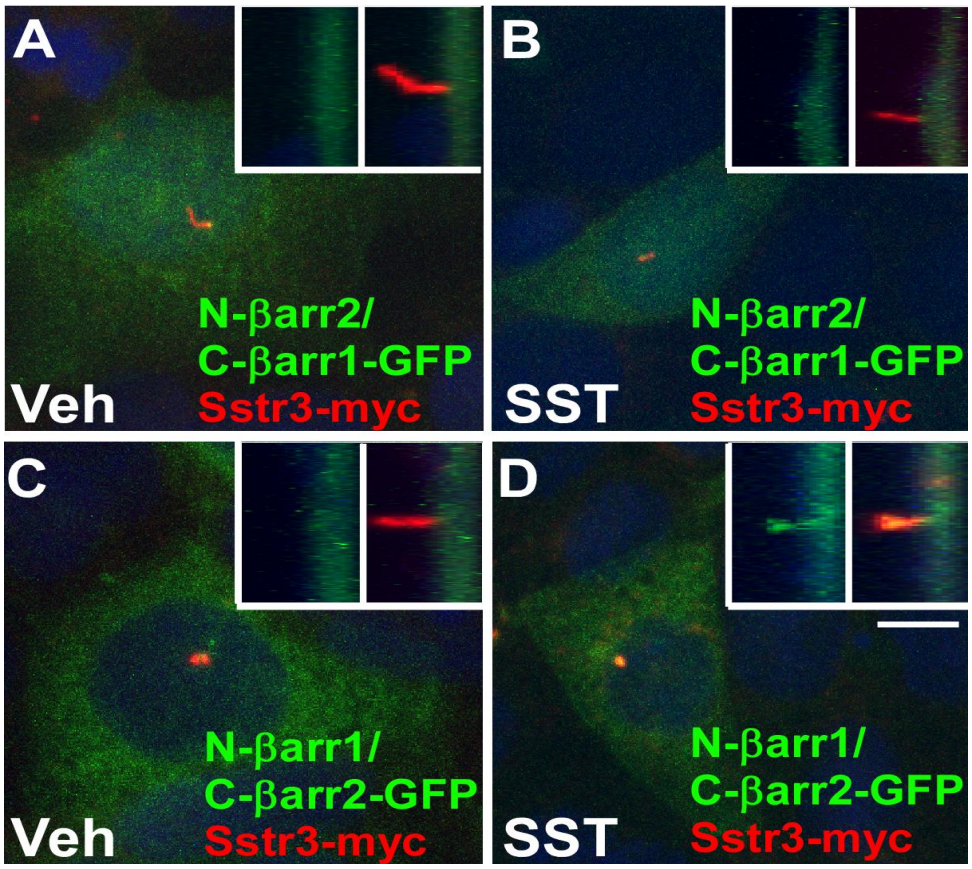

Figure 12 Recruitment of β-arrestin Half Chimeras.

IMCD cells co-transfected with βarr chimera 1 or 2-GFP and Sstr3-myc. Cells were vehicle (Veh) or somatostatin (SST) treated. A&B are chimera 2 (βarr2 1-202 – βarr1 201-418). C&D are chimera 1 (βarr1 1-200- βarr2 203-410). Only chimera 1, which contains the second half of βarr2, is recruited to the primary cilium following treatment with SST. The second half of βarr2 is mediating its recruitment. Scale bar = 5 μm.

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